PTO 03-3027 Japanese Kokai Patent Application No. Sho 61[1986]-17

MUCOPOLYSACCHARIDE-TYPE CANCER-METASTASIS INHIBITOR

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UNITED STATES PATENT AND TRADEMARK OFFICE
WASHINGTON, D.C. MAY 2003
TRANSLATED BY THE RALPH MCELROY TRANSLATION COMPANY

JAPANESE PATENT OFFICE
PATENT JOURNAL (A)
KOKAI PATENT APPLICATION NO. SHO 61[1986]-17

Int. Cl.[4]: A 61 K 31/725
// C 08 B 37/00

Sequence Nos. for Office Use: 6664 – 4C
7133 – 4C

Filing No.: Sho 59[1984]-118283

Filing Date: June 11, 1984

Publication Date: January 6, 1986

No. of Inventions: 1 (Total of 13 pages)

Examination Request: Not filed

MUCOPOLYSACCHARIDE-TYPE CANCER-METASTASIS INHIBITOR

[Muko-tatoukei Gan Teni Yokuseizai]

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[There are no amendments to this patent.]

Claim /1*

A mucopolysaccharide-type cancer-metastasis inhibitor characterized by containing hyaluronic acid, crosslinked hyaluronic acid or their salt as an active component.

Detailed explanation of the invention

This invention pertains to a mucopolysaccharide-type cancer-metastasis inhibitor.

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* [Numbers in the margin represent pagination of original text.]

...cer treatments, there have been surgeries, radiation therapies and chemotherapies mostly ...ted, but no treatment effects satisfactory with respect to cancer recurrence and life-exten... effects have been achieved yet.

One of the causes is that these treatment methods can cause shrinkage or removal or the primary foci of cancers, but the cancers metastasize and proliferate to sites other than the primary foci, especially major organs such as the brain, lungs, liver, etc., with fatal results. Therefore, it is extremely important to prevent cancer metastasis in addition to therapies to attempt to reduce the size of primary foci or remove cancers surgically in order to eradicate cancers.

The process of metastasis of tumor cells comprises many courses such as (a) rapid cell proliferation at the onset site, (b) invasion into the blood vessels, (c) deposition inside capillaries of specific organs, (d) permeation from the inside to the outside of blood vessels, (e) rapid proliferation at the site of metastasis, etc. In principle, the metastasis can be inhibited if it is possible to inhibit any of these courses. There are techniques of histological and biochemical quantitative determination for the courses from (c) to (e), that is, deposition on the inner wall of the blood vessel, subsequent permeation to the outside and proliferation by injecting a constant number of tumor cells directly into the vein of a mouse and observing the behavior of the cells with time and number of metastatic colonies newly regenerated inside the target organs, and many examples have been reported.

For example, Raz, et al. (Raz, et al.; Cancer Research, **40**, 1645-1651 (1980)) injected 50,000 mouse melanoma cells inside the vein of a C57BL/6 mouse, extracted the lung after 18 days and counted the number of black colonies of melanoma cells generated as a result of metastasis. As a result, they reported that the mean number of colonies was found to be closely related to the cell surface chemical composition of melanoma cells. /2

Furthermore, the course of tumor cells depositing on the blood vessel endothelia is absolutely necessary in the case of metastasis of such tumor cells, and this deposition has been found to be induced as a result of mutual recognition and bonding between the molecule distributed on the tumor cell surface and that comprising blood vessel endothelia from the results of many basic experiments (R.H. Kramer, et al.; Proceedings of National Academy of Science, U.S.A., **76**, 5704-5708 (1979)).

On the other hand, Honma, et al. (T. Honma, et al.; Gann, **72**, 898-905 (1981)) have proven that the higher the ability of metastasis of FM3A cells, the shorter the survival life of the host mice. Furthermore, Kimata, et al. (K. Kimata, et al.; Cancer Research, **43**, 1347-1354 (1983)) reported that the higher the ability of metastasis of FM3A cells, the higher the amount of hyaluronic acid (abbreviated to "HA" below) found on the cell surface. In general, HA has been found to show affinity to HA receptors on the cell membrane surface as well as fibronectin and collagen present on the cell surface and various tissues and organs of the body.

Furthermore, HA at a certain concentration level has been known to inhibit phagocytosis of macrophages (E.A. Balazs; Immunology, **40**, 435-448 (1980)) and at a very low concentration level, it has been known to increase, *in vitro* and *in vivo*, the amount of motion, metabolic speed and phagocytosis of macrophages and polynuclear leukocytes (PMN) (L. Häkansson, et al.; Scand. J. Immunol., **11**, 649-653 (1980)).

However, there has been no report on the application of HA as a cancer-metastasis inhibitor.

Furthermore, polysulfated polysaccharides have been reported to have anticancer effects and cancer-metastasis inhibitory actions (Eiro Tsubura, et al.; Gann, **67**, 849-856 (1976); Keiichi Suematu, et al.; Gann, **62**, 331-338 (1971); and Shigeyoshi Anzai; Nichiidaishi, **47** (5), 497-504 (1980)), but the effects observed are mostly attributable to the anti-blood coagulation action and fibrinolysis action of those polysulfated polysaccharides, and HA has hardly any of these actions.

Therefore, the inventors of this invention presumed that if HA was administered to an animal, it was bonded to an HA receptor on the blood vessel endothelia or fibronectin appeared on the surface preventing the cancer cells from being deposited on the endothelia of blood vessels, in addition freeing the once-deposited cancer cells from the endothelia by antagonistically competing with them and at the same time, inhibiting the cancer cells by using the immunological enhancement action of HA; they studied diligently, and as a result, they arrived at the present invention.

Specifically, the mucopolysaccharide-type cancer-metastasis inhibitor of this invention contains HA, crosslinked HA or their salt as an active component.

This invention is explained further in detail as follows.

The HA of this invention is the one from an umbilical cord, crista galli, vitreous body, etc., and the origin is not especially limited. The molecular weight is generally in the range of several thousands to several millions. As a purification process, there are methods described in Japanese Kokai Patent Application Nos. Sho 52[1977]-145594, Sho 52[1977]-105199, Sho 54[1979]-67100 and Sho 55[1980]-74796.

The crosslinked HA of this invention is a crosslinked HA prepared by crosslinking HA or its salt with a polyfunctional epoxy compound, the number of crosslinks is at least 5 per 1000 units of repeating disaccharide of HA comprising glucuronic acid and N-acetylglucosamine (called "repeating disaccharide of HA," below), and it is described in detail in Japanese Patent Application No. Sho 59[1984]-88440.

The polyfunctional epoxy compound of this invention is a compound having at least one epoxy group, and in addition, it is a compound having one or more functional group including an epoxy group suitable for crosslinking HA.

As a specific example of such a compound, there are, for example, halomethyloxirane compounds, bisepoxy compounds, etc. As a halomethyloxirane compound, there are epichlorohydrin, epibromohydrin, β-methylepichlorohydrin, β-methylepibromohydrin, etc. As a bisepoxy compound, there are 1,2-bis(2,3-epoxypropoxy)ethane, 1,4-bis(2,3-epoxypropoxy)butane, 1,6-bis(2,3-epoxypropoxy)hexane, diglycidyl ether of bisphenol A or bisphenol F, etc.

As a salt of HA or crosslinked HA, there are salts of alkali metals such as sodium, potassium, etc.; salts of alkaline-earth metals such as calcium, magnesium, etc.; etc.

The crosslinked HA has hyaluronidase resistance, and it can be synthesized as follows.

In general, HA or its salt having a molecular weight of several thousands to several millions is dissolved in an alkaline aqueous solution at a concentration of over 0.5%, preferably over 1.0%, and a water-soluble organic solvent is added at a concentration of 30% or higher, preferably 50% or higher to the total amount of liquid. The alkaline aqueous solution has a pH of 8-14, preferably 12-14. As a base used in this case, there are generally metal hydroxides such as sodium hydroxide, potassium hydroxide, calcium hydroxide, etc., and metal carbonates such as sodium carbonate, potassium carbonate, etc. As a water-soluble organic solvent, there are methanol, ethanol, isopropanol, acetone, dioxane, etc. These solvents may be used alone or as a mixture. The reaction can be carried out effectively by adding these water-soluble organic solvents, and furthermore, the decomposition of HA (degradation to low-molecular weight fragments) by the base can also be inhibited.

Subsequently, one or more polyfunctional epoxy compounds selected from those described above are added to the solution prepared as described above, and the reaction is carried out at a temperature in the range of 0-100°C, preferably 10-80°C and optimally 20-40°C. The reaction time depends on the reaction temperature used, but if the reaction is carried out at about 20°C, the reaction is preferably carried out from 24 to 48 h, and if the reaction temperature is around 40°C, the reaction time is in the range of 2-3 h.

In this reaction, the crosslinking rate of the cross-linked HA or its salt to be prepared can be adjusted by changing the molar ratio between HA or its salt and polyfunctional epoxy compound.

To obtain the crosslinking HA of this invention having a number of crosslinks of 5 per 1000 repeating disaccharide units of HA, 1 mol of the polyfunctional epoxy compound is used for 1 mol of the repeating disaccharide of HA. If the amount of the polyfunctional epoxy compound to be used per mole of the repeating disaccharide of HA is in the range of 1-10 mol in the case of HA having a molecular weight of about one million, it is possible to obtain a water-soluble and thread-forming crosslinked HA (called "s-crosslinked HA," below), and if the amount of moles used is 10 mol or higher, it is possible to obtain a water-insoluble and gel-state

cross-linked HA (called "is-crosslinked HA," below). Furthermore, in the case of HA having a molecular weight of about 2 million, the same objective can be accomplished by using 2-6 mol and 6 mol or more, respectively.

The s-crosslinked HA is highly viscous, that is, the viscosity is higher than that of HA, the viscosity (at 20°C and shear rate of 1.0 sec^{-1}) of 1% solution in a physiological saline solution is generally in the range of 850-50,000 centipoise, and nonNewtonian index (Hitoshi Kondo, Kitazato Igaku, **10**, 485 (1980)) is in the range of 0.5-0.8.

The crosslinked HA and its salt show resistance against hyaluronidase, and at the same time, they maintain various characteristics of HA.

Especially, s-crosslinked HA is water-soluble, at the same time, it can pass through an injection needle in spite of high viscosity, and consequently, it is preferably used in this invention.

Furthermore, as an HA material used in the cancer-metastasis inhibitor of this invention, the intrinsic viscosity is preferably in the range of 0.2-30, that is, the molecular weight is preferably in the range of 4000-2,000,000.

In the case of applications of the cancer-metastasis inhibitor of this invention, various formulation types such as granules, particles, powder, tablet, capsule, syrup, suspension, liquid, etc., or as it is in the stock powder form may be used for oral administration, and alternatively as an injection formulation, intravenous injection, intra-arterial injection, portal vein administration, thoracic or abdominal cavity administration, intramuscular injection, subcutaneous injection or administration in the tumor itself may be carried out. Furthermore, in the case of formulations such as suppositories, etc., per-rectum administration or nonoral administration is possible. It is possible to use medical organic or inorganic and solid or liquid carriers or diluents suitable for /4
oral, per-rectum or nonoral administration for preparation of the formulations of the cancer-metastasis inhibitor of this invention. Specifically, as a carrier for HA usable in this invention, there are water, gelatin, lactose, starch, magnesium stearate, talc, animal or plant fats and oils, benzyl alcohol, gum, polyalkylene glycol, petroleum resin, coconut oil, lanolin and all other carriers usable in medical drugs. In addition, stabilizers, wetting agents, emulsifiers and salts to change osmotic pressure or maintain a desirable pH level of the formulations may be suitably used as an auxiliary drug component.

The clinical dose depends on the molecular weight of the HA used, but in general in the case of oral administration to adults, the administration of 25 mg to 5 g a day as HA or crosslinked HA is suitable, and the dose is suitably increased or reduced depending on age, morbid state and symptoms. The cancer-metastasis inhibitor in the above daily dose is administered once a day or 2 or 3 proportions with a suitable interval between administrations, and it is also possible to use intermittent administration.

Furthermore, in the case of injection formulations, a single dose of 10 mg to 2.5 g as HA or crosslinked HA may be administered to an adult continuously or intermittently.

The cancer-metastasis inhibitor of this invention shows none of those adverse reactions such as bone marrow disorder, cardiac toxicity, alopecia, etc., observed in conventional anticancer drugs such as alkylation agents, metabolic antagonists, etc., and it has an analgesic action as well as action repairing tissue damage caused by inflammation. Furthermore, there is an advantage of hardly any interactions with those effective drug components suitably usable with the cancer-metastasis inhibitor of this invention such as conventional antimalignant tumor drugs, anti-inflammation drugs, antibiotics, hemostatic agents, gastric ulcer-treatment drugs, etc.

The cancer-metastasis inhibitor of this invention is used for inhibition of metastases of various malignant tumors actively synthesizing proteoglycan and having it on the cell surface considering its pharmacology, and its effects are expected to be excellent especially in highly metastatic malignant tumors such as malignant melanoma, fibrosarcoma, lymphosarcoma, lymphoma, etc. Furthermore, its excellent effects are expected at the time of surgical therapies when the metastasis is liable to occur.

This invention is explained specifically in detail by using preparation, test and application examples as follows, but these examples do not restrict the scope of this invention at all.

Incidentally in the following examples, the measurements of intrinsic viscosity, uronic acid (glucuronic acid) content, nitrogen content and protein content, antigenicity test, exothermic substance tests and bacterial tests were carried out according to the methods described in "Pharmacopoeia japonica 10," general testing method, Item 28 viscosity measurement method; Z. Dische, J. Biol. Chem., **167**, 189 (1947); "Pharmacopoeia japonica 10," general testing method, Item 25 nitrogen quantitative determination method; O.H. Lowry, et al., J. Biol. Chem., **193**, 265 (1951); "Pharmacopoeia japonica 10," dextran 40 injection solution; "Pharmacopoeia japonica 10," general testing method, Item 30 exothermic substance testing method; and Nippon Pharmacology Association, ed., "Hygienic testing methods: 1.4 Microbiological testing methods" (1980), respectively.

Preparation Example 1 Extraction and purification of HA

To 1.0 kg of thawed crista galli cut off from chicken heads and frozen immediately, 3 L of a 0.06% aqueous cetylpyridinium chloride solution were added, and after holding at 95°C for 3 h, the crista galli was removed. After grinding, 3 L water were added, 200 thousand units of Prolysine (trade name of protease manufactured by Ueda Kagaku Kogyo K.K.) were added, and after maintaining the mixture at 50°C for 5 h, it was filtered to obtain 3400 mL filtrate. To 3400 mL of the filtrate, 170 g sodium chloride were added and dissolved, subsequently, 3500 mL 95% ethanol were added; the precipitates formed were collected and dried to obtain 6.1 g HA.

Furthermore, the HA prepared was dissolved in a sterilized physiological saline solution so that the concentration was 1%, and conventional procedures such as sterilization filtration, etc. were carried out to obtain an HA solution in a physiological saline solution.

The physical properties of the HA powder and HA solution in a physiological saline solution prepared are as follows.

HA powder (Sample No. HA-1)

Intrinsic viscosity: 26.5

Uronic acid content: 48.4%

Nitrogen content: 3.48%

Protein content: 0.01%

Antigenicity: none

1% solution in a physiological saline solution

HA concentration: 1.00%

Exothermic substance: none

No. of microorganisms: bacteria 0 cell/g

fungi 0 cell/g

Preparation Example 2 Extraction and purification of HA

The same HA preparation procedures as those in Preparation Example 1 were carried out for 10 kg of thawed crista galli cut off from chicken heads and frozen immediately. The physical properties of the HA powder and HA solution in a physiological saline solution prepared were as follows.

HA powder yield: 62.0 g (Sample No. HA-2)

Intrinsic viscosity: 15.0

Uronic acid content: 48.7%

Nitrogen content: 3.46%

Protein content: 0.018%

Antigenicity: none

1% solution in a physiological saline solution

HA concentration: 0.99%

Exothermic substance: none

No. of microorganisms: bacteria 0 cell/g

fungi 0 cell/g

Preparation Example 3 HA preparation

In 0.1M acetic acid buffer solution (pH 5.0), 10 g of the sample No. HA-2 were dissolved to obtain a 1.0% solution, 9 mg bovine testicular hyaluronidase (manufactured by Seikagaku Kogyo) were added, and the reaction was carried out at 50°C for 1.3 h. To the reaction solution obtained, 1.5 times equivalent of ethanol were added to obtain precipitates, which were subsequently dissolved again in purified water to obtain an HA concentration of 2%, and 1.5 times equivalent of ethanol were added again to obtain precipitates. The precipitates were dissolved in purified water so that the HA concentration was 2%, sterilized active carbon (heated at 121°C for 60 min and washed with purified water) was added, and the mixture was filtered by using sterilized radiolite (heated at 121°C for 60 min and washed with purified water). Ethanol was added to the filtrate to obtain precipitates.

The physical properties of the HA powder and HA solution in a physiological saline solution prepared were as follows.

HA powder yield: 6.7 g (Sample No. HA-3)

Intrinsic viscosity:	7.0
Uronic acid content:	48.0%
Nitrogen content:	3.47%
Protein content:	0.010%
Antigenicity:	none

1% solution in a physiological saline solution

HA concentration:	1.02%	
Exothermic substance:	none	
No. of microorganisms:	bacteria	0 cell/g
	fungi	0 cell/g

Preparation Example 4 Preparation of HA

The same procedures as those used in Preparation Example 3 were carried out by using the sample No. HA-2 to obtain HA products shown in the following table.

Table 1

Sample No.	HA powder (Sample No. HA-1)					1% solution in a physiological saline solution			
	Intrinsic viscosity	Uronic acid content	Nitrogen content	Protein content	Antigenicity	HA concentration	Exothermic substance	No. of microorganisms	
								Bacteria	Fungi
HA-4	2.5	48.50	3.44	0.009	—	1.02	—	0	0
HA-5	0.8	48.40	3.52	0.012	—	1.00	—	0	0
HA-6	0.4	48.70	3.48	0.010	—	1.04	—	0	0

Preparation Example 5 Preparation of IA /6

In 0.1M acetic acid buffer solution (pH 5.0), 10 g of the sample No. HA-2 were dissolved to obtain a 1% solution, 100 mg bovine testicular hyaluronidase were added, and the reaction was carried out at 50°C for 37 h. The reaction mixture obtained was concentrated under vacuum, desalted by using a Sephadex G10 column and fractionated to a fraction containing an octasaccharide or higher saccharides and that containing saccharides lower than an octasaccharide by using a Sephadex G25 column. The fraction containing saccharides lower than an octasaccharide was desalted by using a Sephadex G10 column, concentrated under vacuum, and after filtration sterilization, it was freeze-dried.

The physical properties of HA powder prepared (Sample No. HA-7) were as follows.

Intrinsic viscosity: 0.075
Uronic acid content: 48.28%
Nitrogen content: 3.48%
Protein content: 0.011%
Antigenicity: none

Thin-layer chromatography: saccharides smaller than an octasaccharide (Merck, Quizel [transliteration] Gel 60F, developing solvent: n-propanol:conc. aqueous ammonia:water (40:30:2.5), color development: anisealdehyde-sulfuric acid

Preparation Example 6

(1) Synthesis of s-crosslinked HA

In 450 mL of a 0.2N aqueous sodium hydroxide solution, 10 g of HA sodium salt (molecular weight of 7.3×10^5) were dissolved while cooling, and the mixture was filtered through a 0.45 µm microfilter. To the filtrate obtained, 40 mL of a 10N aqueous sodium hydroxide solution were added, and while stirring, 500 mL ethanol and 6.0 mL epichlorohydrin were added. The reaction was carried out at 20°C for 24 h, and the reaction mixture solution was adjusted to pH 6.4 with acetic acid. Subsequently, 500 mL ethanol were added to obtain white

precipitates, which were collected by filtration, washed thoroughly with ethanol and dried under vacuum.

Yield: 8.9 g (Sample No. s-crosslinked HA-1)

No. of crosslinks per 1000 repeating disaccharide units of HA: 8.5

Viscosity in 1% solution in a physiological saline solution: 11,000 centipoise (20°C, shearing rate of 1.0 sec^{-1})

NonNewtonian index: 0.60

Elemental analysis C: 42.0%, H: 4.87%, N: 3.29%, Na: 5.81%

(2) Gel chromatography for s-crosslinked HA

Gel chromatography was carried out for the s-crosslinked HA synthesized in (1) and HA used for the synthesis by using a column (6 x 850 mm) packed with glass beads CPG 3000 (Electro Nucleonics, Inc.). The developing solvent used was a 1.5M aqueous sodium chloride solution adjusted to pH 8.5 with sodium hydroxide, the eluate was fractionated to 0.52 mL each, and uronic acid was quantitatively determined by using the carbazole-sulfuric acid method. The results obtained are shown in Figure 1. In Figure 1, the empty circle O and filled circle ● show the results on the absorbance in the carbazole-sulfuric acid method determined for respective fractions of the s-crosslinked HA and HA, respectively, and V_o shows the gel particle external volume.

As apparent from the results shown in Figure 1, the s-crosslinked HA is found to be a very high-molecular-weight compound compared with HA.

(3) NonNewtonian index of s-crosslinked HA

The nonNewtonian index (m = a/b) was calculated for 1% solutions of the s-crosslinked HA synthesized in (1) and HA used for the synthesis in a physiological saline solution by using a rotary viscometer (Tokyo Keiki K.K., type E viscometer) and carrying out measurements at 37°C while changing the shearing speed. The results obtained are shown in Figure 2. In Figure 2, the empty circle O and filled circle ● show the results on the viscosity of 1% solutions of the s-crosslinked HA and HA in a physiological saline solution, respectively at various shearing speed levels.

(4) Thread-forming property of s-crosslinked HA

The thread-forming properties of the s-crosslinked HA synthesized in (1) and HA used for the synthesis were evaluated by using a device constructed by using a Watanabe-type thread-forming property measurement device (Hiroshi Ikeuchi, Nippon Seikeigeka Gakkaishi, **34**, 175 (1980)) as a model. The results obtained are shown in Figure 3. In Figure 3, the empty

circle O, empty triangle Δ and filled circle ● show the results on thread formation at resp... pull-up speeds measured for 0.5% solution of the s-crosslinked HA in a physiological sali... solution, 1% solution of the same and 1% solution of HA in a physiological saline solutio... respectively.

As apparent from the results shown in Figure 3, the s-crosslinked HA has a high thread-forming property.

(5) Analgesic effect of s-crosslinked HA

The analgesic effect of s-crosslinked HA synthesized in (1) was evaluated as follows.

Beagles were used without any discrimination in their sex. As a pain-causing substance, 20 μg bradykinin or 2 mg acetylcholine were administered with or without a 2.5 mg/0.5 mL /7
solution of s-crosslinked HA in a physiological saline solution to the knee joint of one hind leg, and the variation of a load on the hind leg of drug administration was measured with time. As a reference, a 5 mg/0.5 mL solution of the sodium HA used as the raw material in (1) in a physiological saline solution was used. The analgesic effect was compared with the results on the time required for 50% recovery to the normal load. The results obtained are shown in Table 2.

Table 2

Pain-causing substance	50% recovery time
Bradykinin	8.6 min
Bradykinin + HA-Na	3.4 min
Bradykinin + s-crosslinked HA	4.0 min
Acetylcholine	21 min
Acetylcholine + HA-Na	11 min
Acetylcholine + s-crosslinked HA	11 min

As apparent from the results shown in Table 2, the s-crosslinked HA shows an excellent analgesic effect as that of sodium HA.

Preparation Example 7 Synthesis of s-crosslinked HA

To a 1% aqueous potassium HA solution (molecular weight of 1.7×10^6), 0.1 mL 10N potassium hydroxide and 5 mL methanol were added. While stirring, 17 mg epibromohydrin were added, and the reaction was carried out at 20°C for 24 h. Subsequently, the reaction mixture was adjusted to pH 6.5 with acetic acid, and 10 mL ethanol were added to obtain white precipitates, which were collected by filtration and dried under vacuum.

Yield: 98 mg

No. of crosslinks per 1000 repeating disaccharide units of HA: 7.5

Viscosity in 1% solution in a physiological saline solution: 34,000 centipoise (20°C, shearing rate of 1.0 sec^{-1})

NonNewtonian index: 0.65

Elemental analysis C: 41.98%, H: 4.79%, N: 3.30%, K: 9.45%

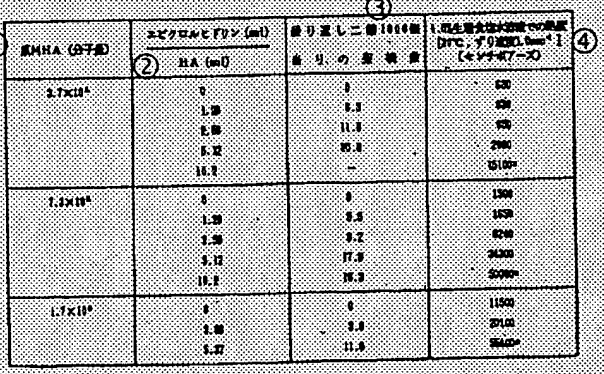
Preparation Example 8 Crosslinking rate of crosslinked HA

To a solution of 100 mg of sodium HA having a molecular weight of 3.7×10^5 or 7.3×10^5 dissolved in 5.0 mL 1N sodium hydroxide, 5 mL ethanol and 25, 50, 100 or 200 μL epichlorohydrin were added, and the reaction was carried out at 40°C for 2 h. The same post-treatment procedures as those in Preparation Example 6 (1) were carried out after the reaction was completed.

Furthermore, to a solution of 75 mg sodium HA having a molecular weight of 1.7×10^6 dissolved in 7.5 mL 1N sodium hydroxide, 7.5 mL ethanol and 40 or 80 μL epichlorohydrin were added, and the reaction was carried out at 40°C for 2 h. The reaction was also carried out under the same conditions as those described above by using [2-^{14}C]-epichlorohydrin (obtained from Amersham, Japan Co.), and from the radioactivity of the labeled compound, the crosslinking rate was calculated. Table 3 shows the relationship between the crosslinking rate and viscosity.

As apparent from the results shown in Table 3, the crosslinking rate and viscosity in s-crosslinked HA are found to have a proportional relation.

Table 3



* If it goes over this value, the solution becomes a gel (insoluble in water).

Key: 1 Raw material HA (molecular weight)

2 Epichlorohydrin (mole)/HA (mole)
3 Number of crosslinks per 1000 repeating disaccharide units
4 Viscosity of 1.0% solution in a physiological saline solution (at 20°C, shearing speed of 1.0 sec^{-1}) (centipoise)

Test Example 1 Hyaluronidase resistance of s-crosslinked HA /8

The following 3 kinds of s-crosslinked HA compounds were prepared from the raw material sodium HA having a molecular weight of 7.3×10^5 by using the same procedures as those used in Preparation Example 6 (1).

(A) No. of crosslinks per 1000 repeating disaccharide units of HA: 13
Viscosity in 1% solution in a physiological saline solution: 45,500 centipoise
(20°C, shearing rate of 1.0 sec^{-1})
NonNewtonian index: 0.77

(B) No. of crosslinks per 1,000 repeating disaccharide units of HA: 11.5
Viscosity in 1% solution in a physiological saline solution: 28,000 centipoise
(20°C, shearing rate of 1.0 sec^{-1})
NonNewtonian index: 0.70

(C) No. of crosslinks per 1000 repeating disaccharide units of HA: 7.5
Viscosity in 1% solution in a physiological saline solution: 8000 centipoise
(20°C, shearing rate of 1.0 sec^{-1})
NonNewtonian index: 0.61

The viscosity was measured (20°C and shearing speed of 1.0 sec^{-1}) for those 3 kinds of s-crosslinked HA's and sodium HA used for the syntheses dissolved respectively in a 0.1M aqueous acetic acid solution (pH 5.0) at a concentration of 1%, and the results as follows were obtained.

s-Crosslinked HA (A) 45,000 centipoise

s-Crosslinked HA (B) 27,000 centipoise
s-Crosslinked HA (C) 8000 centipoise
sodium HA 1500 centipoise

To those solutions, 0.09 wt% bovine testicular hyaluronidase was added, the reaction was carried out at 50°C, the viscosity was measured after 15, 35, 55 and 70 min. and the proportion to the viscosity before the reaction was calculated.

The results obtained are shown in Figure 4. In Figure 4, the empty square □, empty triangle Δ, empty circle O and filled circle ● show the results on the proportion of viscosity at respective reaction time to the viscosity before reaction for those s-crosslinked HA (A), (B) and (C) and sodium HA, respectively.

As apparent from the results shown in Figure 4, the s-crosslinked HA's used in this invention show a higher resistance against hyaluronidase than that of HA, and the higher the degree of crosslinking, the more remarkable the resistance.

Test Example 2 Effects on proliferation ability of tumor cells

A cultivation medium containing 0.15 mL of one of various HA solutions was mixed with 1.5 mL of a cell suspension (Eagle MEM cultivation medium with 10% of calf serum) of 1 x 10^4 cells/mL of FM3A/p-15A cell, and the cultivation was carried out in a tissue culture Petri dish (Telmo Co. Petray 12F) under conditions of 5% CO_2-95% air at 37°C. The number of cells was measured after 3 and 5 days from the start of cultivation. The experiments were carried out with 4 dishes per group, and in the control group, a physiological saline solution was added to the cultivation medium.

The results obtained are shown in Table 4.

Table 4

⑥	①	②	③				
			④				⑤
⑦ 3日	HA-B	200	7.6	14	11	13	11.4±2.8
		1000	13	9	14	9	11.7±3.7
	HA-C	100	13	8	13	12	9.3±1.5
		1000	9	10	10	10.5	11.8±1.1
	⑨対照群		12	12.8	10.5	11.9	11.8±1.1
⑧ 5日	HA-B	100	25	25	25	31	26.2±2.7
		1000	26.5	31.5	28	25	25.7±2.8
	HA-C	100	24	20	22	21	22.5±1.7
		1000	20	21	22	20	22.3±1.3
	⑨対照群		20	20	21.5	17	19.4±1.3

Key: 1 Experimental group
2 No. of cells (x 10^4 cells/mL)

3 Day after HA concentration (μg/mL)
4 Results of measurement
5 Mean±standard deviation
6 Starting cultivation
7 3 days
8 5 days
9 Control group

As apparent from the results shown in Table 4, the presence of HA did not affect the cell proliferation. /9

Test Example 3 Effects on bonding ability of tumor cells

FM3A/p-15A cells cultivated in a 100-mm Falcon tissue culture dish was washed with Dulbecco's phosphate buffer solution (free of Ca and Mg) (abbreviated to "PBS(-)," below) and treated with a solution of 0.1% trypsin and 0.04% ethylenediamine tetraacetate dissolved in a Hank's buffered salt solution (free of Ca and Mg) (abbreviated to "TE," below) at 37°C for 5 min. The same amount of a cultivation solution (10% of bovine fetal serum added to Eagle's MEM (minimum essential medium) cultivation medium) was added, the mixture was centrifuged at 1200 rpm for 5 min, and the same cultivation solution was used to adjust to 5 x 10^5 cells/mL (treated cell A).

On the other hand, FM3A/p-15A cells cultivated in a 100-mm Petri dish was centrifuged at 1200 rpm for 5 min, and the above cultivation solution was used to adjust to 5 x 10^5 cells/mL (treated cell B).

In a 35-mm culture dish coated with collagen (abbreviated to "CoI," below), fibronectin (abbreviated to "FN," below) or laminin (abbreviated to "LN," below), 1 mL each of the treated cell A and treated cell B were placed, and it was adjusted to 1 x 10^6 cells/culture dish. Various HA's were added at a concentration of 1 mg/mL, the cultivation was carried out at 37°C for 20 h, subsequently, the culture was washed with PBS(-), treated with TE at 37°C for 15 min, and the number of cells was measured. The results obtained are shown in Table 5.

Table 5

① 基質 \ HA	②HA-2 粘度15.0 分子量94万 ③	②HA-6 粘度 0.4 分子量8000 ④	②HA-7 粘度 0.675 分子量1500 ④	対⑤照
Co I	103	91	83	84
FN	88	53	85	81
LN	7	2	68	79

Key: 1 Control

2 Intrinsic viscosity Intrinsic viscosity 0.4 Intrinsic viscosity 0.075
3 Molecular weight 840,000
4 Molecular weight
5 Substrate

As apparent from the results shown in Table 5, the cancer-metastasis inhibitor of this invention markedly reduced the bonding ability of the tumor cells, and the effects were found to be more remarkable in HA-2 with intrinsic viscosity of 15.0 (molecular weight of 840,000) and HA-6 with intrinsic viscosity of 0.4 (molecular weight of 8000) than in HA-7 with intrinsic viscosity of 0.075 (molecular weight of 1500).

Test Example 4 Acute toxicity test

(1) Table 6 shows the number of deaths and results on LD_{50} with time after administration of HA-2 to mice.

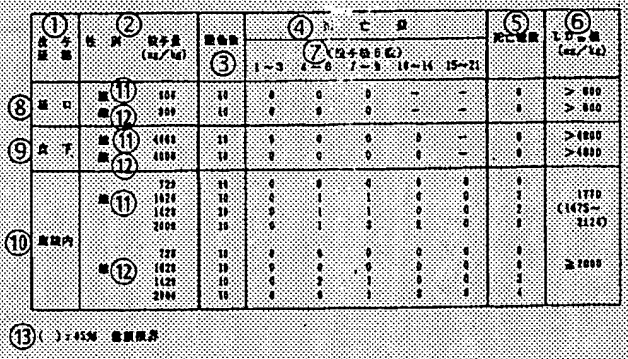
Table 6

①	②	③	④ 死亡数 ⑥(投与後日数)				⑤	⑤A
			1～3	4～6	7～9	10～14	死亡総数	LD_{50}値 (mg/kg)
⑦	⑧ 2100	10	0	0	0	-	0	>2100
	⑨ 2100	10	0	0	0	-	0	>2100
⑩	⑧ 4000	10	0	0	0	0	0	>4000
	⑨ 4000	10	0	0	0	0	0	>4000
⑪	⑧ 720	10	0	0	0	0	0	>2000
	1420	10	0	0	0	0	0	
	1420	10	0	0	0	0	0	
	2000	10	0	1	0	0	1	
	⑨ 720	10	0	0	0	0	0	≧2000
	1420	10	0	0	0	0	0	
	1420	10	0	1	0	0	1	
	2000	10	0	1	1	0	2	

Key: 1 Administration path
2 Sex, Dose (mg/kg)
3 No. of animal
4 No. of deaths
5 Total No. of death
5A LD_{50} results
6 (No. of days after administration)
7 Oral
8 Male
9 Female
10 Subcutaneous
11 Abdominal cavity

(2) Table 7 shows the number of deaths and results on LD_{50} with time after /10
administration of HA-2 to rats.

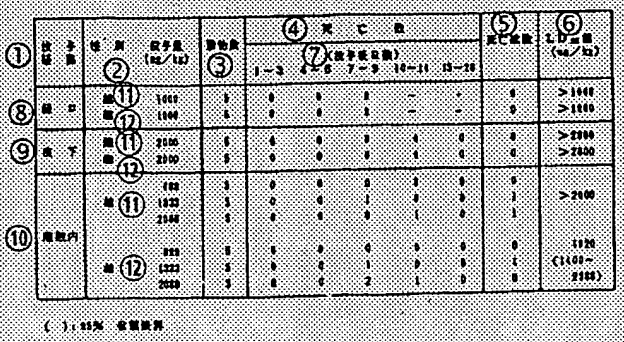
Table 7



Key:
1 Administration path
2 Sex, Dose (mg/kg)
3 No. of animals
4 No. of deaths
5 Total No. of deaths
6 LD_{50} results
7 (No. of day after administration)
8 Oral
9 Subcutaneous
10 Abdominal cavity
11 Male
12 Female
13 ( ): 95% confidence limit

(3) Table 8 shows the number of deaths and results on LD_{50} with time after administration of HA-2 to rabbits.

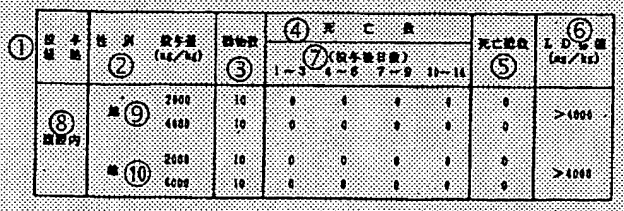
Table 8



Key:
1 Administration path
2 Sex, Dose (mg/kg)
3 No. of animal
4 No. of deaths
5 Total No. of deaths
6 LD_{50} results
7 (No. of day after administration)
8 Oral
9 Subcutaneous
10 Abdominal cavity
11 Male
12 Female
13 ( ): 95% confidence limit

(4) Table 9 shows the number of deaths and results on LD_{50} with time after administration of HA-6 to mice. /11

Table 9



Key:
1 Administration path
2 Sex, Dose (mg/kg)
3 No. of animal
4 No. of deaths
5 Total No. of deaths
6 LD_{50} results
7 (No. of day after administration)

8 Abdominal cavity
9 Male
10 Female

Application Example Cancer-metastasis inhibitory effects of HA and s-crosslinked HA

The abdominal cavity administration was carried out on C3H/He mice for solutions of various HA's having different intrinsic viscosity levels and s-crosslinked HA in a physiological saline solution, and after 30 min, 7.5 x 10^5 cells of highly metastatic cancer cells, FM3A/p-15A of mouse breast cancer-origin were injected into the mouse tail vein.

After 3 h from the injection, the 1st abdominal cavity administration of a solution of HA or s-crosslinked HA in a physiological saline solution, and including this 1st administration, the abdominal cavity administration of a solution of HA or s-crosslinked HA in a physiological saline solution was carried out 2 times a day for a total of 4 days.

The mice were slaughtered after 21 days from the cancer cell administration, the lungs were extracted, and the number of metastatic foci of the cancer was counted.

Incidentally, in the control group, a physiological saline solution alone was administered instead of HA or s-crosslinked HA. The results obtained are shown in Table 10.

Table 10

①	②	③	④	⑤ ⑥	⑦
HA-7	0.475	20 40	20 20	26.8 74.8	85.8 82.2
HA-6	0.4	10 20	10 10	23.1 24.0	23.0 20.9
HA-5	0.8	10 20	10 10	20.8 19.3	87.9 74.1
HA-4	2.6	0.01 0.10 1.00	10 10 10	20.3 15.8 10.4	20.0 17.4 13.0
HA-3	7.0	0.25	10	13.0	17.1
HA-2	15.0	0.25 0.50	20 10	2.2 0.4	2.8 11.0
HA-1	20.0	0.25	10	2.0	2.5
⑧ ナトリウム塩 HA-1	19.0	0.25	10	6.2	8.9
⑨ 対照群			40	80.0	100

Key: 1 Experimental group
2 No. of animals
3 No. of metastatic foci in the lung
4 Intrinsic viscosity
5 Dose (mg/mouse/day)
6 Mean

7 [illegible]entage to the control group
8 [illegible]oss-linked HA-1
9 [illegible]trol group

As apparent from the results shown in Table 10, the cancer-metastasis inhibitor of this invention is found to have excellent cancer-metastasis inhibitory effects. /12

Brief description of the drawings

Figure 1 is a drawing showing gel chromatograms of the s-crosslinked HA and HA. Figure 2 is a drawing showing the results of viscosity measurements carried out for the s-crosslinked HA and HA. Figure 3 is a drawing showing the results of thread-forming property measurements carried out for the s-crosslinked HA and HA. Figure 4 shows a drawing showing the relationship between viscosity reduction and time in the case of treatment of various s-crosslinked HA's and HA with hyaluronidase.

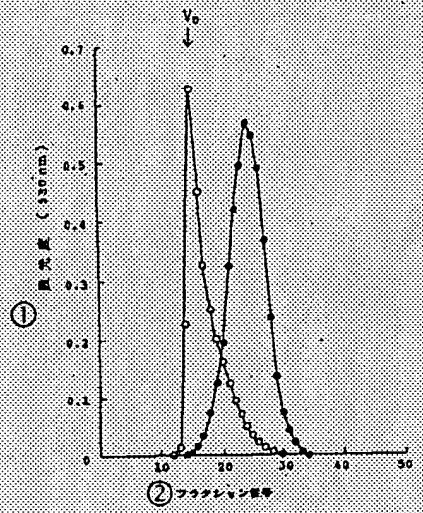


Figure 1

Key: 1 Absorbance
2 Fraction number

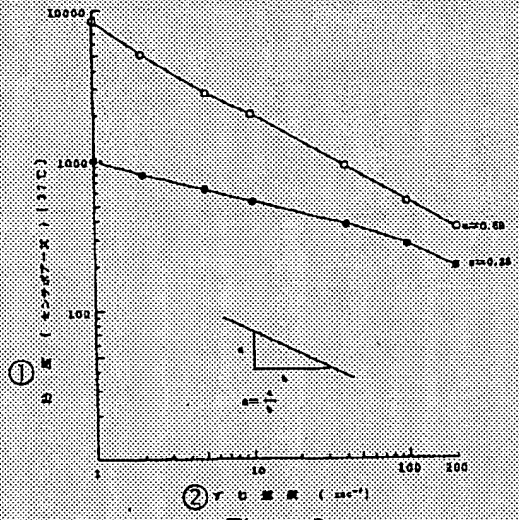


Figure 2

Key: 1 Viscosity (centipoise) (37°C)
2 Shearing speed (sec^{-1})

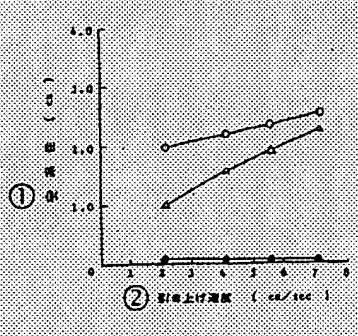


Figure 3

Key: 1 Thread-forming property
2 Pull-up speed (cm/sec)

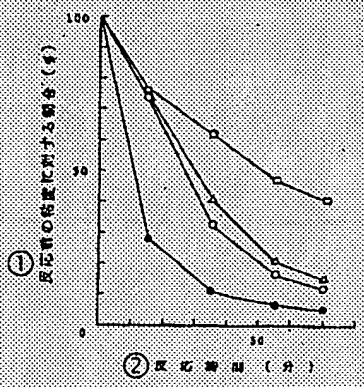


Figure 4

Key: 1 Proportion to viscosity before reaction (%)
2 Reaction time (min)